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Expression Cloning of a Human B_1 Bradykinin Receptor*

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A cDNA clone encoding a human B_1 bradykinin receptor was isolated from a human embryonic lung fibroblast cDNA library by expression cloning. The photoprotein aequorin was utilized as an indicator of the ability of the B_1 receptor agonist [des-Arg^{10}]kallidin to mediate Ca^{2+} mobilization in *Xenopus laevis* oocytes injected with RNA. A clone was isolated with a 1307-nucleotide insert which contains an open reading frame encoding a 353-amino acid protein with the characteristics of a G-protein-coupled receptor. The amino acid sequence of the B_1 bradykinin receptor is 36% identical to the amino acid sequence of the B_2 bradykinin receptor. The cloned B_1 bradykinin receptor expressed in mammalian cells exhibits high affinity binding for ^{3}H-labeled [des-Arg^{10}]kallidin and low affinity for bradykinin. The B_1 receptor antagonist [des-Arg^{10},Leu^9]kallidin effectively displaces ^{3}H-labeled [des-Arg^{10}]kallidin from the cloned receptor, whereas the B_2 receptor antagonist Hoe-140 (D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin, where Thi is L-[3-(2-thienyl)alanyl], Tic is D-(1,2,3,4-tetrahydroisoquinolin-3-yl-carbonyl), and Oic is L-[(3a*S*, 7a*S*)-octahydroindol-2-yl-carbonyl]) does not. Therefore, the expressed receptor has the pharmacological characteristics of the B_1 receptor subtype. The availability of both the cloned human B_1 and B_2 bradykinin receptors should allow the elucidation of the relative contributions of these two receptor subtypes in acute and chronic inflammatory processes.

Two mammalian bradykinin receptor subtypes, B_1 and B_2, have been defined based on their pharmacological properties (1, 2). The B_1 receptor is synthesized *de novo* following tissue injury and has recently been shown to mediate hyperalgesia in animal models of chronic inflammation (1). The B_2 bradykinin receptor is normally present in smooth muscle and certain neurons, where activation of B_2 receptors causes pronounced hypotension, bronchoconstriction, pain, and inflammation (1, 2). The agonists for the B_1 and B_2 bradykinin receptors are generated by the proteolytic action of kallikreins which release the nonapeptide bradykinin (BK)[1] and the decapeptide Lys-BK (kallidin) from large protein precursors, low and high molecular weight kininogen. BK and kallidin are equipotent agonists at the B_2 receptor. In contrast, BK is inactive at the B_1 bradykinin receptor subtype. Degradation of the B_2 receptor agonists by a carboxypeptidase produces the B_1 receptor agonists, [des-Arg^9]BK and [des-Arg^{10}]kallidin. The phenomenon of proteolytic transformation of a peptide from B_2 to B_1 selectivity has been observed not only for the endogenous kinin agonists but also for several synthetic peptide antagonists (3, 4).

The B_1 receptor was originally discovered through a contractile response to [des-Arg^9]BK that was observed in rabbit aortic strips only after a prolonged *in vitro* incubation (5–7). The *de novo* synthesis of B_1 receptors has been reported *in vivo* following treatment with bacterial lipopolysaccharide (8) and in animal models of antigen arthritis (9). *In vitro* studies have implicated a number of cytokines, most notably interleukin-1 (IL-1) and IL-2, as mediators that induce the expression of B_1 receptors (6, 10–12). Furthermore, the activation of a B_1 bradykinin receptor on mouse macrophages causes the release of cytokines (13, 14). Significantly, the B_1 bradykinin receptor antagonist [des-Arg^9,Leu^8]BK was recently found to alleviate hyperalgesia in animal models of persistent inflammation (1, 15, 16). Thus, a body of evidence implicates the B_1 bradykinin receptor in the pathophysiology of chronic inflammation. Relatively little is known about the role of the B_1 receptor in healthy tissues, although both B_1 and B_2 receptors may play a physiological role in renal function (17, 18).

The cloning of the B_2 bradykinin receptor has revealed that this receptor is a member of the superfamily of G-protein-coupled receptors (19–22), definitive evidence that the B_1 receptor couples to G-proteins has not been forthcoming. The rat B_2 bradykinin receptor was cloned (19) using a *Xenopus* oocyte expression system that exploited the ability of the B_2 receptor to act through G-proteins to activate phospholipase C and mobilize Ca^{2+} (23, 24). Recently, the B_1 bradykinin receptor has also been shown to activate phospholipase C in primary cultures of rabbit aorta smooth muscle cells, rabbit mesenteric artery smooth muscle cells, and rat mesangial cells (25–27). Furthermore, both B_1 and B_2 bradykinin receptor activities were detected when mRNA from the human fibroblast cell line WI-38 was injected into *X. laevis* oocytes (28, 29). Although the similarity of ligands for the two bradykinin receptor subtypes suggests a similarity between the B_1 and B_2 receptor genes, the results of genomic Southern analyses indicated that these two receptors are not highly homologous (19, 30). Therefore, to clone the human B_1 receptor, we pursued an expression cloning strategy in *Xenopus* oocytes utilizing the photoprotein aequorin as an indicator of Ca^{2+} mobilization (31, 32). We isolated a cDNA clone that encodes a G-protein-coupled receptor with an amino acid sequence that is 36% identical to that of the B_2 bradykinin receptor. The pharmacological properties of this cloned receptor expressed in mammalian cells demonstrate that it is a B_1 bradykinin receptor.

MATERIALS AND METHODS

Oocyte Injections—Injection of mRNA or cRNA into *Xenopus* oocytes was performed by a modification of established protocols (33, 34). The excised ovarian lobes were teased apart with jeweler's forceps and then placed into OR-2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U12512.

[1] The abbreviations used are: BK, bradykinin; IL, interleukin; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase(s).

HEPES, pH 7.4) containing 2 mg/ml collagenase B (Boehringer Mannheim) for 2 h at room temperature. Oocytes were selected and cultured overnight in supplemented OR-2 (OR-2 containing 1.8 mM $CaCl_2$, 0.5 mg/ml gentamycin, and 0.5 mM theophylline). Initially, oocytes were injected with 46 nl of RNA at a concentration of 1 or 2 mg/ml in H_2O. Once the pool size became less than 30 clones, the cRNA concentration was decreased to 40 ng/ml. RNA was injected using a Nanoject automatic oocyte injector (Drummond Scientific), and injection needles were pulled from 3.5-inch Drummond capillaries using a Flaming/Brown Micropipette puller (Sutter Instruments). Two to three days after the RNA injection, oocytes were injected with 92 ng of aequorin (Friday Harbor Photoproteins) resuspended in 46 nl of 1 mM EDTA, as described previously (31, 32). The following day, individual oocytes placed in wells of a microtiter dish containing 225 µl OR-2 were challenged with peptide agonists, and the aequorin photo response was measured using a ML3000 microtiter plate luminometer (Dynatech).

RNA Fractionation—IMR-90 cells (ATCC CCL 186) were grown in minimal essential medium supplemented with 10% fetal calf serum, glutamine, nonessential amino acids, sodium pyruvate, penicillin, and streptomycin (Life Technologies, Inc.). Two and a half hours prior to mRNA extraction, IMR-90 cells were exposed to 200 pg/ml IL-1β (R & D Systems). mRNA was purified from these cells using the poly(A) tract mRNA isolation system (Promega) and resuspended in H_2O at a concentration of 2 mg/ml.

IMR-90 mRNA was size-fractionated on a continuous 6–20% sucrose gradient in 15 mM PIPES, pH 6.5, 5 mM EDTA, and 0.25% *N*-lauroylsarcosine. 480 µg of mRNA from the IL-1β-induced IMR-90 cells was heat-denatured and size-fractionated by centrifugation at 18 °C for 19 h at 77,000 × *g*. Fractions (450 µl) from each gradient were collected from the bottom of the tube. Fractions were ethanol-precipitated twice and resuspended to a final concentration of 1 µg/µl. RNA size determination was based on the migration pattern of 80 µg of 9.49–0.24-kb RNA markers (Life Technologies, Inc.) loaded on a parallel gradient.

Library Construction—First strand cDNA synthesis of 3 µg of approximately 1.7-kb size-selected mRNA was primed with 50 ng of random hexamers and 400 ng of a *Not*I oligo(dT) oligonucleotide and synthesized with the Life Technologies, Inc. Superscript II reverse transcriptase. Following second strand synthesis, *Bst*XI/*Eco*RI adaptors (Invitrogen) were ligated onto the ends, and the cDNA was passed over a Life Technologies, Inc. cDNA sizing column. The cDNA was cloned into pcDNA3 (Invitrogen) cut with *Bst*XI. Plasmid DNA was transformed into XL-1 Blue cells (Stratagene).

Colonies were plated on Colony/Plaque screen filters (DuPont NEN) that were placed on Luria-Bertani (LB) agar plates supplemented with 100 µg/ml ampicillin (Sigma). Plasmid DNA was linearized with *Not*I, and cRNA was synthesized using T7 RNA polymerase with the mCAP RNA capping kit (Stratagene).

The DNA sequence of both strands of clone 33E9 was determined by a combination of manual sequencing using Sequenase version 2.0 (U. S. Biochemical Corp.) and automated sequencing using an ABI 373A (Perkin-Elmer).

Mammalian Cell Expression and Pharmacological Characterization—COS-7 cells were transfected by electroporation using a Bio-Rad gene pulser. Three days post-transfection, cells were processed for either whole cell or membrane binding assays as described previously (27, 35). Displacement studies were done with 1 nM [des-Arg^{10}],[3,4-^{3}H]kallidin (DuPont NEN) in the presence of varying concentrations of competitor compounds. Binding assays were performed at room temperature for 45 min. Reactions were terminated by filtration using either an Inotech cell harvester or a Tomtech cell harvester onto glass fiber filters that had been briefly soaked in 0.3% polyethyleneimine. The filters were washed with cold phosphate-buffered saline and counted either in an LKB Betaplate 1205 or a Beckman liquid scintillation counter.

RESULTS AND DISCUSSION

The human embryonic fibroblast cell line IMR-90 had been shown previously to express the B_1 bradykinin receptor subtype (36). A more detailed pharmacological characterization revealed the presence of approximately 5000 high affinity binding sites for the B_1 agonist ^{3}H-labeled [des-Arg^{10}]kallidin and approximately 70,000 high affinity binding sites for the B_2 agonist [^{3}H]BK per cell (data not shown). Furthermore, treatment of IMR-90 cells with the cytokine IL-1β was found to stimulate the number of B_1 bradykinin receptors approximately 7-fold. The ability of the B_1 receptor expressed in IMR-90 cells to mobilize Ca^{2+} in response to [des-Arg^{10}]kallidin was demonstrated using Fura-2 as an indicator.[2] Based on these data, we chose IL-1β-induced IMR-90 cells as the source of mRNA for expression cloning.

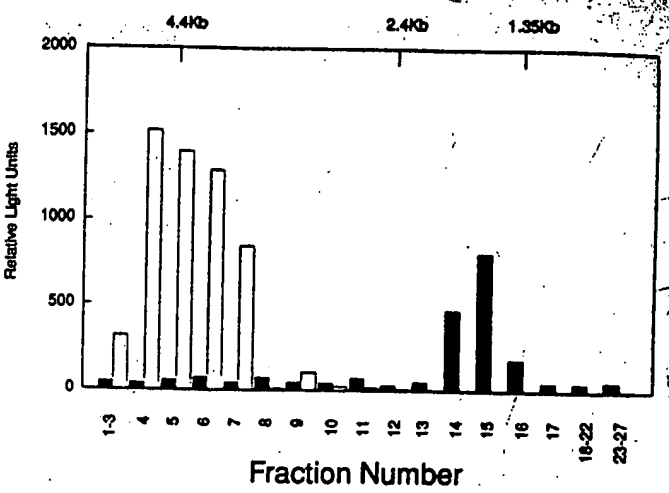


FIG. 1. **Sucrose-gradient size fractionation of IL-1β-induced IMR-90 mRNA.** Fractionation was performed as described under "Materials and Methods." Oocytes were injected with equal volumes of mRNA and challenged with bradykinin agonists. The Ca^{2+} response to 100 nM bradykinin (*open bars*) or 20 nM [des-Arg^{10}]kallidin (*closed bars*) is presented in relative light units. Each value is the mean response of five oocytes.

Injection of mRNA prepared from IL-1β-induced IMR-90 cells into *X. laevis* oocytes resulted in aequorin-mediated luminescence in response to either the B_1 agonist [des-Arg^{10}]kallidin or the B_2 agonist BK. The mRNA was size-fractionated over a sucrose gradient, fractions were injected into oocytes, and the oocytes were assayed for their ability to respond to either BK or [des-Arg^{10}]kallidin. The B_1 and B_2 receptor transcripts were clearly separated by the size fractionation (Fig. 1). The mRNA mediating the response to [des-Arg^{10}]kallidin exhibited an apparent size of 1.6–1.8 kb, whereas the mRNA mediating the response to BK had an apparent size of 4.4–4.6 kb. The apparent size of the mRNA enabling the BK response is consistent with the previously determined size of the B_2 bradykinin receptor transcript (19, 30).

The RNA fraction from the sucrose gradient which gave the greatest response to [des-Arg^{10}]kallidin was utilized to generate a cDNA library. The library contained greater than 90% inserts, with an average insert size of 1.9 kb. The library was plated in pools of approximately 5000 clones that were used to synthesize cRNA. Of the 25 pools of cRNA that were injected into *Xenopus* oocytes, 11 exhibited aequorin-mediated luminescence in response to [des-Arg^{10}]kallidin. The pool that gave the most robust response was replated and fractionated into 25 pools of approximately 800 clones. Eight pools exhibited a response to [des-Arg^{10}]kallidin. The strongest positive pool was further examined using electrophysiology to monitor activation of the Ca^{2+} activated Cl^- channel (data not shown). [des-Arg^{10}]Kallidin (20 nM) produced a response that was blocked by prior incubation with the B_1 receptor antagonist [des-Arg^{10},Leu^9]kallidin (20 nM). This pool was then subdivided into 32 pools of approximately 25 individual clones. Two positive pools, containing 14 and 34 clones, respectively, were identified. cRNA was prepared from individual clones and analyzed in *Xenopus* oocytes. Three individual clones were found to elicit a [des-Arg^{10}]kallidin response. One clone, 33E9 (Fig. 2), was chosen for further DNA sequence analysis, expression, and pharmacological characterization.

[2] R. W. Ransom, unpublished observations.

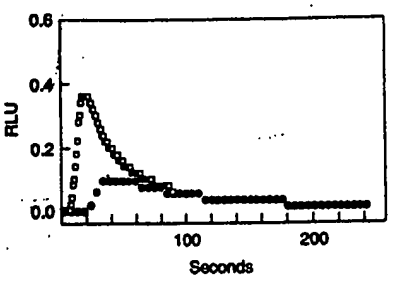


FIG. 2. **Functional response of clone 33E9 in *Xenopus* oocytes.** Luminometer tracing of the response to 20 nM [des-Arg^{10}]kallidin of an individual *Xenopus* oocyte injected with IMR 90 mRNA (*closed circles*) or cRNA prepared from clone 33E9 (*open squares*). The B_1 receptor agonist was added at time 0 and the aequorin response measured as described under "Materials and Methods."

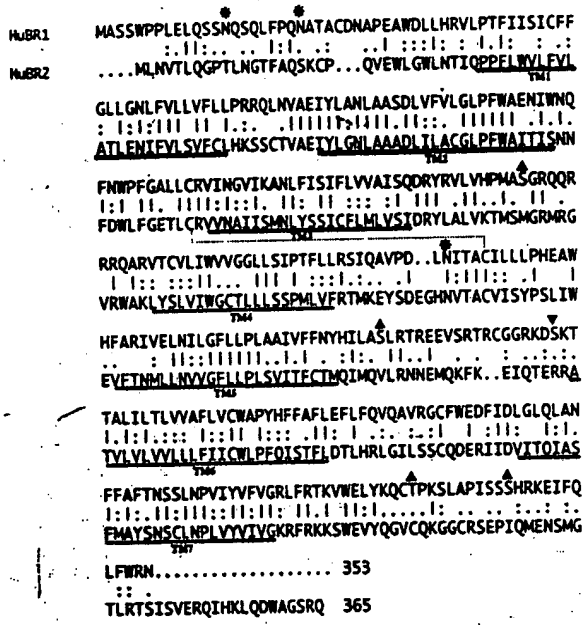
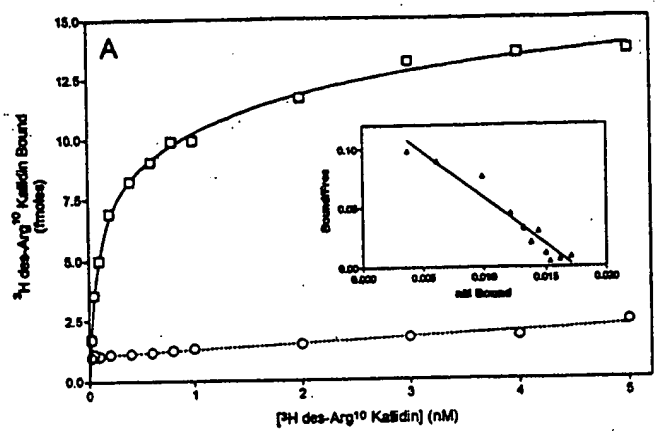


FIG. 3. **Comparison of the amino acid sequence of the human B_1 bradykinin receptor and the human B_2 bradykinin receptor.** The alignment was performed using the GAP program in the GCG software package. The seven putative transmembrane domains are *underlined*. The symbol * indicates a potential *N*-linked glycosylation site, ▲ indicates potential protein kinase C phosphorylation sites, and ▼ indicates potential cAMP-dependent protein kinase sites. The highly conserved cysteine residues that are proposed to be involved in a disulfide bond are connected by a *dotted line*.



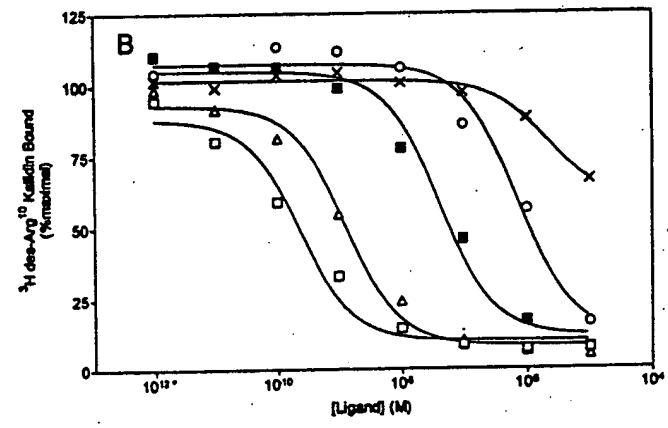


FIG. 4. **Pharmacological properties of the human B_1 bradykinin receptor expressed in COS-7 cells.** *A*, specific binding (*open squares*) of increasing concentrations of ^{3}H-labeled [des-Arg^{10}]kallidin to membranes from COS-7 cells transfected with the B_1 bradykinin receptor cDNA. Nonspecific binding is shown by the *open circles*. A Scatchard transformation of the specific binding data is shown (*inset*). *B*, displacement of 1 nM ^{3}H-labeled [des-Arg^{10}]kallidin from COS-7 cells expressing the human B_1 bradykinin receptor by increasing concentrations of compounds. Symbols for compounds: □, [des-Arg^{10}] kallidin; △, [des-Arg^{10},Leu^9]kallidin; ■, kallidin; ○, [des-Arg^9]BK; and ×, bradykinin.

Clone 33E9 contains an insert of 1307 nucleotides with an open reading frame of 1059 nucleotides. We isolated several different clones that encompassed the same DNA sequence as clone 33E9 but began and ended at different locations, indicating that they were independently derived. The sequence surrounding the proposed initiator methionine codon at nucleotide 209 conforms to the Kozak consensus sequence in the +4 position but not at the −3 position (37). The open reading frame encodes a protein that is 36% identical to the B_2 bradykinin receptor (Fig. 3). The sequence identity at the nucleotide level, 54%, probably explains the failure to clone this receptor by low stringency hybridization with DNA encoding the B_2 receptor. A homology search of the Swiss Protein data base indicates that the B_1 receptor is 30% identical to the angiotensin type 2 receptor and 29% identical to the angi tensin type 1 receptor (38–40) and less homologous to other G-protein-coupled receptors. A Kyte and Doolittle hydrophobicity plot of the amino acid sequence reveals the potential for the seven transmembrane domains that are characteristic of G-protein-coupled receptors (41, 42). Two conserved Cys residues that are proposed to form a disulfide bond between the second and third extracellular domains in nearly all G-protein-coupled receptors are also present in this sequence (Fig. 3). There are two consensus sites for *N*-linked glycosylation in the NH_2-terminal domain and one in the third extracellular domain. Potential phosphorylation sites for protein kinase C and cAMP-dependent protein kinase are present in intracellular domains 2 and 3 and the carboxyl-terminal domain. Similar potential phosphorylation sites in other G-protein-coupled receptors have been implicated in short term desensitization of the receptor following agonist stimulation (41, 42).

Clone 33E9 was transfected into COS-7 cells, and the pharmacological properties of the expressed receptor were determined. Scatchard analysis of saturation binding data with ^{3}H-labeled [des-Arg^{10}]kallidin indicated a K_d of 0.4 nM and a B_{max} of approximately 100 fm l/mg of protein (Fig. 4*A*). Mock-transfected COS-7 cells did n t demonstrate any specific binding f r ^{3}H-labeled [des-Arg^{10}]kallidin (data not shown).

The ability of several bradykinin receptor agonists to displace ^{3}H-labeled [des-Arg^{10}]kallidin from the cloned receptor was assessed (Fig. 4*B*, Table I). The IC_{50} for displacement of 1 nM ^{3}H-labeled [des-Arg^{10}]kallidin from the cloned receptor by

TABLE I

Comparison of the peptide binding properties of the cloned human B_1 bradykinin receptor with the B_1 receptor in IMR-90 cells

The IC_{50} was determined by the displacement of 1 nM ^{3}H-labeled [des-Arg^{10}]kallidin in the membrane binding assay described under "Materials and Methods." The data presented are the average of three experiments.

Peptide	IC_{50}, human B_1	IC_{50}, IMR 90
	nM	
[des-Arg^{10}]Kallidin	0.2	0.5
[des-Arg^{10}][Leu^9]Kallidin	1.3	1.3
Kallidin	42	62
Bradykinin	2000	7800
[des-Arg^9]BK	720	590
[des-Arg^9, Leu^8]BK	440	130
Hoe-140	>10,000	>10,000
[des-Arg^{10}]Hoe-140	60	20
[Met,Lys]BK	70	95

BK is >2 μM. The low affinity of this receptor for BK and high affinity for [des-Arg^{10}]kallidin argues strongly that this cloned bradykinin receptor is of the B_1 receptor subtype. Competition binding studies yielded a rank order of affinity for kinin agonists of: [des-Arg^{10}]kallidin > kallidin > [des-Arg^9]BK >> BK at the cloned human receptor. This is very similar to the rank order of potency reported for the rabbit B_1 bradykinin receptor and identical to that observed for the B_1 receptor in IMR-90 cells (Table I).

Both the cloned human B_1 receptor and the B_1 receptor in IMR-90 cells exhibit a relatively low affinity for the "classical" B_1 receptor agonist [des-Arg^9]BK, which has an affinity of 42 nM for the B_1 receptor in rabbit aorta (27). The B_1 bradykinin receptor present in human aorta also has a relatively low affinity of 1 μM for [des-Arg^9]BK.[2] However, like the cloned human B_1, the B_1 receptor in rabbit aorta has a lower affinity for [des-Arg^9]BK than [des-Arg^{10}]kallidin (7, 27). Thus the most potent natural ligand for the B_1 receptor appears to be [des-Arg^{10}]kallidin. Based on the pharmacological profile outlined in Table I, we believe it is likely that the B_1 receptor isolated here is the human homolog of the B_1 receptor present in rabbit aorta and that the lower affinity of the human receptor for [des-Arg^9]BK may be a consequence of species differences.

The ability of several bradykinin receptor antagonists to displace 1 nM ^{3}H-labeled [des-Arg^{10}]kallidin from the cloned receptor was also analyzed. The cloned B_1 receptor has relatively high affinity binding for the B_1-specific antagonists [des-Arg^{10},Leu^9]kallidin and [des-Arg^9,Leu^8]BK (Table I). By contrast, the cloned receptor has a very low affinity for the potent B_2-specific antagonist Hoe-140. Significantly, the removal of the COOH-terminal Arg from Hoe-140 results in a dramatic increase in affinity (Table I), as would be expected for a B_1 receptor (3). Therefore, the interaction of the cloned receptor with bradykinin antagonists is consistent with the B_1 receptor subtype classification.

In summary, we have utilized an expression cloning strategy to isolate a clone encoding a human B_1 bradykinin receptor. This receptor was isolated by its ability, when expressed in *Xenopus* oocytes, to functionally respond to the B_1 receptor agonist [des-Arg^{10}]kallidin. Th cloned receptor is a G-protein-coupled receptor that is most similar in amino acid sequ nce to the B_2 bradykinin receptor. The pharmacological properties of the cloned receptor expressed in mammalian cells are characteristic of the B_1 bradykinin receptor classification. Th B_1 bradykinin receptor has been implicated in chronic inflammation and hyperalgesia, wh reas the B_2 receptor appears to mediate acute inflammatory and algesic responses. Th availability of cl ned human B_1 and B_2 receptors should lead to a greater understanding of the role f these receptors in both normal and pathophysi logical conditions.

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